Exhibit II

Shimamoto et al., "*Characterization of the tritium-labeled analog of L-threo-beta-benzyloxyaspartate binding to glutamate transporters*," Mol. Pharmacol. 71: 294-302 (2007)

0026-895X/07/7101-294–302$20.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 71:294–302, 2007

Vol. 71, No. 1
27250/3164691
Printed in U.S.A.

Characterization of the Tritium-Labeled Analog of L-*threo*-β-Benzyloxyaspartate Binding to Glutamate Transporters

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Received May 29, 2006; accepted October 17, 2006

ABSTRACT

L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. Termination of glutamate receptor activation and maintenance of low extracellular glutamate concentrations are primarily achieved by glutamate transporters (excitatory amino acid transporters 1–5, EAATs1–5) located on both the nerve endings and the surrounding glial cells. To identify the physiological roles of each subtype, subtype-selective EAAT ligands are required. In this study, we developed a binding assay system to characterize EAAT ligands for all EAAT subtypes. We recently synthesized novel analogs of *threo*-β-benzyloxyaspartate (TBOA) and reported that they blocked glutamate uptake by EAATs 1–5 much more potently than TBOA. The strong inhibitory activity of the TBOA analogs suggested that they would be suitable to use as radioisotope-labeled ligands, and we therefore synthesized a tritiated derivative of (2*S*,3*S*)-3-{3-[4-ethylbenzoylamino]benzyloxy}aspartate ([^{3}H]ETB-TBOA). [^{3}H]ETB-TBOA showed significant high-affinity specific binding to EAAT-transfected COS-1 cell membranes with each EAAT subtype. The Hill coefficient for the Na^+-dependence of [^{3}H]ETB-TBOA binding revealed a single class of noncooperative binding sites for Na^+, suggesting that Na^+ binding in the ligand binding step is different from Na^+ binding in the substrate uptake process. The binding was displaced by known substrates and blockers. The rank order of inhibition by these compounds was consistent with glutamate uptake assay results reported previously. Thus, the [^{3}H]ETB-TBOA binding assay will be useful to screen novel EAAT ligands for all EAAT subtypes.

Glutamate acts as a major excitatory neurotransmitter in the mammalian central nervous system and has been implicated in higher brain function and in brain pathophysiology. Excitatory amino acid transporters (EAATs) play important roles in maintaining the extracellular glutamate concentration at low levels to limit the activation of glutamate receptors and to protect neurons from excitotoxicity (Danbolt, 2001). Five subtypes of EAATs have been identified. EAAT1 (GLAST) is expressed in astrocytes throughout the brain, including the cerebellum. EAAT2 (GLT-1) is the most prevalent subtype and is expressed primarily in astrocytes in the forebrain. These two glial transporters are responsible for the majority of glutamate uptake and are critical to the maintenance of glutamate homeostasis in brain. EAAT3 (EAAC1) and EAAT4 are found at postsynaptic nerve endings. EAAT3 is widely distributed in the brain, whereas EAAT4 specifically localizes to the cerebellum (Purkinje cells). EAAT5 is a photoreceptor and bipolar cell glutamate transporter selectively expressed in the retina.

The uptake of glutamate by EAATs is driven by electro-

This work was supported in part by Grants-in-Aid for Creative Scientific Research 13NP0401 (to K.S.) and the Special Coordination Funds for Promoting Science and Technology Target-Oriented Brain Science Research Program (to T.N.), Grants-in-Aid for Scientific Research 15500278 (to Y.S.), 16510174 (to K.S.), 18613006 (to T.N.), and a Grant-in-Aid for Young Scientists 16790051 (to T.N.) from the Ministry of Education, Science, Sports, and Culture of Japan.

K.S. and T.N. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.027250.

ABBREVIATIONS: EAAT, excitatory amino acid transporter; TBOA, *threo*-β-benzyloxyaspartate; ETB-TBOA, (2*S*,3*S*)-3-{3-[4-ethylbenzoylamino]benzyloxy}aspartate; TFB-TBOA, (2*S*,3*S*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate; PMB-TBOA, (2*S*,3*S*)-3-{3-[4-methoxybenzoylamino]benzyloxy}aspartate; CCG-II, (2*S*,1′*R*,2′*R*)-2-(2-carboxycyclopropyl)glycine; CCG-III, (2*S*,1′*S*,2′*R*)-2-(2-carboxycyclopropyl)glycine; iGluR, ionotropic glutamate receptor; mGluR, metabotropic glutamate receptor; Vin-BzA-TBOA, (2*S*,3*S*)-3-{3-[4-vinylbenzoylamino]benzyloxy}aspartate; HPLC, high-performance liquid chromatography; TLC, thin layer chromatography; PBS, phosphate-buffered saline; L-TBOA, (2*S*,3*S*)-3-benzyloxyaspartate; DHKA, dihydrokainate; [^{3}H]4MG, [^{3}H](2*S*,4*R*)-4-methylglutamate.

chemical gradients across the plasma membrane. The process also requires Na^+ for substrate binding and K^+ for net transport. It has been proposed that three Na^+ ions and one H^+ are transported into the cell with glutamate, whereas one K^+ ion is transported out (Zerangue and Kavanaugh, 1996; Levy et al., 1998). In addition, an anion conductance that is thermodynamically uncoupled to glutamate transport has been demonstrated (Fairman et al., 1995; Wadiche et al., 1995; Arriza et al., 1997), although this property varies among the EAAT subtypes. EAAT2 does not exhibit this property, but a Cl^- conductance representing more than 95% of the observed steady-state current associated with glutamate uptake is associated with EAAT4 and EAAT5 (Bergles et al., 2002).

Although important roles of EAATs have been elucidated using genetic approaches, genetic manipulations cannot provide information on the dynamics of glutamate homeostasis in response to short-term disruption of uptake systems. Thus, pharmacological intervention using blockers is an indispensable approach for investigating the physiological roles of each subtype (Campiani et al., 2003; Shigeri et al., 2004; Bridges and Esslinger, 2005; Shimamoto and Shigeri, 2006). We have demonstrated that (2*S*,3*S*)-3-benzyloxyaspartate (L-TBOA) is nontransportable for all subtypes of EAATs (Shimamoto et al., 1998; Shigeri et al., 2001). Recently, we synthesized novel TBOA analogs and reported that benzoylamide derivatives of TBOA [(2*S*,3*S*)-3-{3-[4-substituted-benzoylamino]benzyloxy}aspartate] blocked EAATs1–5 much more potently than TBOA in electrophysiological assays using EAATs expressed in *Xenopus laevis* oocytes (Shimamoto et al., 2004). We also found that low nanomolar concentrations of these compounds inhibited glutamate uptake by COS-1 cells expressing EAATs1–3. However, we could not evaluate glutamate uptake by EAAT4 or EAAT5 because the glutamate uptake capacities of these subtypes are much lower than those of EAATs1–3. Moreover, irreversible inhibition by the TBOA derivatives prevented quantitative analysis in the electrophysiological assay. Thus, we could not evaluate the detailed effects of these compounds on EAAT4 or EAAT5. Likewise, only a few inhibitors of EAAT4 and EAAT5 have been characterized in other reports (Fairman et al., 1995; Arriza et al., 1997; Eliasof et al., 2001; Shigeri et al., 2001), whereas most pharmacological studies to develop subtype-selective blockers have only focused on EAATs1–3. Thus, a simple assay system in which the effects of compounds can be quantitatively compared among all EAAT subtypes would be useful for evaluating new compounds and for screening subtype-selective EAAT ligands.

In the present study, we developed a novel binding assay system for EAATs. The TBOA analogs are potent inhibitors of EAATs, making them highly suitable for use as radioisotope-labeled ligands. Therefore, we synthesized a tritium labeled analog of (2*S*,3*S*)-3-{3-[4-ethylbenzoylamino]-benzyloxy}aspartate ([^{3}H]ETB-TBOA). The potency of ETB-TBOA inhibition of EAATs1–3 in the glutamate uptake assay was comparable with that of (2*S*,3*S*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate (TFB-TBOA), the most potent TBOA analog. [^{3}H]ETB-TBOA showed specific binding to rat brain crude membranes and EAAT-transfected COS-1 cell membranes and bound all of the EAAT subtypes with high affinity. The Hill coefficient for the Na^+-dependence of [^{3}H]ETB-TBOA binding revealed a single class of noncooperative binding sites for Na^+, suggesting that Na^+ binding in the ligand binding step is different from Na^+ binding in the substrate uptake process. The binding of [^{3}H]ETB-TBOA was displaced by known substrate-type inhibitors [L-glutamate, L-aspartate, (2*S*,1′*S*,2′*R*)-2-(2-carboxycyclopropyl)glycine (CCG-III), etc.] and blocker-type inhibitors (TBOA, TFB-TBOA, etc.). The rank order of inhibition by these compounds was consistent with results reported previously in the glutamate uptake assay and in electrophysiological studies. This binding assay also enabled us to quantify the responses of EAAT4 and EAAT5. These findings suggest that [^{3}H]ETB-TBOA will serve as a useful tool to screen novel EAAT ligands for all EAAT subtypes.

Materials and Methods

Materials. ETB-TBOA and (2*S*,3*S*)-3-{3-[4-vinylbenzoylamino]-benzyloxy}aspartate (Vin-BzA-TBOA) were synthesized as optically pure forms from a commercially available epoxide, (2*S*,3*R*)-[3-(benzyloxymethyl)oxiranyl]methanol *para*-nitorobenzoate (Fluka Chemie AG, Buchs, Switzerland) in the same manner as TFB-TBOA (Shimamoto et al., 2004). The structure and purity (>95%) of the compounds were confirmed by 400 MHz NMR and HPLC. Stock solutions (10 mM) were prepared in dimethyl sulfoxide as trifluoroacetic acid salts and were stored at −20°C until the day of the experiments. TBOA analogs were confirmed to be stable in the stock solutions for more than 3 months. Tritium-labeled ETB-TBOA ([^{3}H]ETB-TBOA) was prepared by Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan), as shown in Fig. 1. The specific radioactivity was 1.48 TBq/mmol, and the concentration was 37.0 MBq/ml. Chemical and radiochemical purities were confirmed to be >97% by TLC and HPLC. TLC (Silica gel 60 F254; Merck, Darmstadt, Germany) was developed in 1-butanol/acetic acid/water at a ratio of 4:1:2 (v/v), and radioactivity was detected using a bioimaging analyzer (BAS2000; Fuji Photo Film, Tokyo, Japan). HPLC was performed using a Cosmosil 5 C_{18}-MSII column (4.6-mm i.d.× 150 mm; Nacalai Tesque, Kyoto, Japan), and the retention time was 5.40 min [eluent: acetonitrile/water 3:7 (v/v) containing 0.1% trifluoroacetic acid; flow rate: 1.0 ml/min; detection: UV (254 nm)]. The radioactivity of each peak was detected by adding the scintillation cocktail FLO-SCINT II (PerkinElmer Life and Analytical Sciences, Boston, MA). [^{3}H]ETB-TBOA was stored in ethanol at −20°C. Other radiolabeled ligands were purchased from PerkinElmer. All other chemicals were of the highest purity available and were purchased from Nacalai Tesque, Sigma (St. Louis, MO), or Tocris Cookson (Bristol, UK).

Transfection. Eukaryotic expression vectors (pKDEMSS) containing cDNA of EAATs1–3 were prepared as reported previously (Shimamoto et al., 1998). EAAT4 cDNA was obtained from a human cerebellum cDNA library (Clontech, Palo Alto, CA) by polymerase chain reaction using a 5′-upstream primer (5′-ATAGACCATGAGCAGCCATGGCA-3′) and 3′-downstream primer (5′-CTCACATAGCACTCTCGTTGCCT-3′). The products were cloned into pCRII (In-

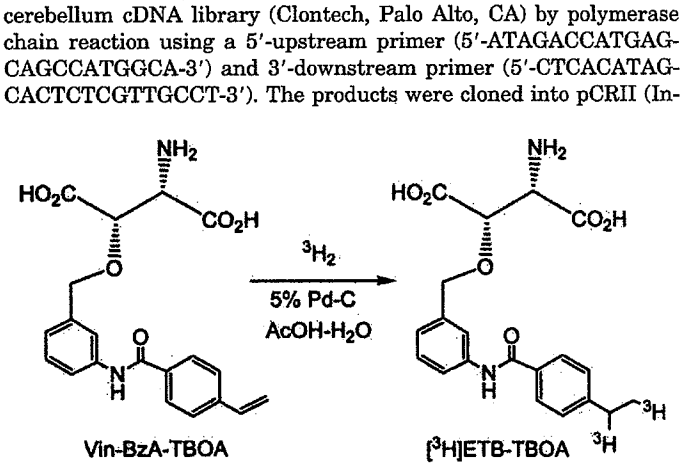

Fig. 1. Synthesis of [^{3}H]ETB-TBOA from Vin-BzA-TBOA.

vitrogen, Carlsbad, CA) and analyzed by DNA sequencing. A clone containing EAAT4 was digested with EcoRI and subcloned into the EcoRI site of pKDEMSS. EAAT5 cDNA was obtained using the following primers for polymerase chain reaction with pOTV-EAAT5 as a template: 5′-upstream primer (5′-GGAATTCCACCATGGTGC-CGCATACCATC-3′) and 3′-downstream primer (5′-GACTAGTCT-CAGACATTGGTCTCCAGCTC-3′). The EcoRI (underlined)-SpeI (bold and underlined) fragment was inserted into the EcoRI-SpeI site of pKDEMSS and analyzed by DNA sequencing. COS-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2. Cells were transfected by electroporation (1×10^7 cells, 200 V, 975 μF) with 10 μg of the vector containing each EAAT subtype. COS-1 cells transfected by the vector alone were used as the control. For the binding assay, transfected cells were cultured for 2 days, harvested using EDTA-trypsin, washed with phosphate-buffered saline (PBS), and stored at −80°C until use.

Membrane Preparation. On the day of the assay, the frozen cells were suspended in binding buffer (120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.6) using a Polytron homogenizer (Kinematica, Basel, Switzerland) (setting 6, 30 s), followed by centrifugation at 20,000*g* for 10 min. The pellet was resuspended in the binding buffer at a final concentration of 100 to 500 μg/ml. To examine ion dependence, several buffers were prepared as follows. NaCl buffer contained 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$. To examine the concentration response for Na^+, various concentrations of NaCl were replaced by the same concentration of choline chloride. In LiCl buffer, ChCl buffer, and KCl buffer, 120 mM LiCl, choline chloride, or KCl, respectively, were substituted for NaCl in the NaCl buffer. For chloride-free buffer, all Cl^- ions in the NaCl buffer were replaced by gluconate, and for potassium-free buffer, KCl in the NaCl buffer was replaced with NaCl. Cell pellets were washed twice with the specific buffer used in the assay before resuspension in the same buffer at the final assay concentration. Rat brain crude membranes were prepared from forebrain as described previously (Murphy et al., 1987; Sakai et al., 2001) and stored at −80°C until use. The membranes were washed with the binding buffer and resuspended at a final concentration of 50 to 100 μg/ml.

Transporter Binding Assay Using Cell Membranes. An aliquot of membranes (200 μl) was incubated with the designated concentration of [^{3}H]ETB-TBOA in the presence or absence of various concentrations of test compounds, in triplicate, for 30 min at room temperature. Nonspecific binding was determined in the presence of 10 μM TFB-TBOA. Bound radioactivity was separated from free radioactivity by filtration through a glass filter (Filtermat A; PerkinElmer) using a Micro Cell Harvester (Skatron Instruments AS, Tranby, Norway). The filter was attached to a solid scintillation cocktail (MeltiLexA; PerkinElmer) and counted using a Betaplate1205 (PerkinElmer). A K_d value for each EAAT subtype was obtained from a Scatchard plot of the data. Dose-response curves were fitted to the Hill equation using Prism III (GraphPad Software Inc., San Diego, CA). IC_{50} values were obtained from the dose-response curves and presented as mean ± S.E.M. from at least three experiments. K_i values were determined from the equation $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is the concentration of [^{3}H]ETB-TBOA. For saturation experiments, binding of [^{3}H]ETB-TBOA was measured over concentrations range of 5 to 100 nM for EAAT1,2,4,5 and 10–300 nM for EAAT3. To generate a Na^+ concentration-response curve, 20 nM [^{3}H]ETB-TBOA was used, and the specific binding at each Na^+ concentration was normalized to the amount bound at 120 mM Na^+ (100%). The Hill coefficient was determined from the slope of a Hill plot of $\log[B/(B_{max} - B)]$ as a function of $\log[Na^+]$.

Measurements of [^{14}C]Glutamate Uptake in Transfected COS-1 Cells. Transfected COS-1 cells or MDCK cells stably expressing EAAT2 or EAAT3 were seeded in 96-well plates and cultured for 2 days. The subconfluent cells were washed two times with 300 μl of modified PBS that contained 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM D-glucose, pH 7.4, and preincubated in 300 μl of the same buffer at 37°C for 12 min. After aspiration of the buffer, cells were incubated with 1 μM L-[^{14}C]glutamate in 100 μl of the modified PBS in the presence or absence of test compounds at various concentrations at 37°C for 12 min. To terminate uptake, cells were washed three times with ice-cold buffer. Radioactivity was measured by scintillation counting (TopCount; PerkinElmer) in 100 μl of MicroScint 40 (PerkinElmer). Nonspecific uptake was determined in the presence of 1 μM TFB-TBOA. Specific uptake of [^{14}C]glutamate is presented relative to the control. All values displayed are the mean ± S.E.M. of at least three experiments.

Results

Synthesis of Tritiated Ligand. In recent years, many groups have reported novel inhibitors for EAATs. However, interactions with all EAAT subtypes have only been characterized for a few of these compounds. For most compounds, only inhibition of EAATs1–3 has been characterized because glutamate uptake by EAAT4 and EAAT5 is not of sufficient magnitude to be evaluated experimentally. We compared [^{14}C]glutamate uptake by COS-1 cells expressing EAATs1–5. The amount of [^{14}C]glutamate uptake by EAATs1–5 was 5.3 ± 0.3-, 5.8 ± 0.4-, 7.5 ± 0.3-, 2.1 ± 0.1-, and 1.5 ± 0.2-fold relative to the control (vector-transfected cells), respectively. Because COS-1 cells express endogenous transporters, evaluation of specific uptake by EAAT4 or EAAT5 in the uptake assay was not possible. Thus, we have developed a novel binding assay system capable of evaluating all EAAT subtypes. Recently, we demonstrated that low nanomolar concentrations of TBOA analogs inhibited glutamate uptake and blocked glutamate-induced transport currents in *X. laevis* oocytes much more potently than TBOA (Shimamoto et al., 2004), without any effects on other receptors and transporters. We synthesized several TBOA analogs as candidates for a radioligand and found that [^{3}H]ETB-TBOA can be easily labeled from the vinyl precursor (Vin-BzA-TBOA) by tritium gas addition in the presence of a palladium catalyst (Fig. 1). Chemical and radiochemical purity of the compound was confirmed to be >97% by silica gel TLC and by HPLC. The IC_{50} values of unlabeled ETB-TBOA in the [^{14}C]glutamate uptake assay using MDCK cells stably expressing EAAT2 or EAAT3 were 3.2 ± 0.3 and 88 ± 4.8 nM, respectively. These values are very similar to IC_{50} values of TFB-TBOA, which is, thus far, the most potent blocker of these transporters [1.9 ± 0.1 nM (EAAT2) and 28 ± 1.8 nM (EAAT3)] (Shimamoto et al., 2004). ETB-TBOA did not show any significant activity in several other assays, including a binding assay for ionotropic glutamate receptors (iGluRs) (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, kainate, and *N*-methyl-D-aspartate subtypes), a Ca^{2+} mobilization assay for metabotropic glutamate receptors (mGluR1 and mGluR5), a cAMP formation inhibition assay (mGluR2 and mGluR4), or a glutamate uptake assay using isolated synaptic vesicles (data not shown). HPLC analysis of unlabeled ETB-TBOA showed that no detectable decomposition occurred after 1 month of storage (10 mM dimethyl sulfoxide solution), even at room temperature. The transporter binding characteristics of [^{3}H]ETB-TBOA did not decrease after 1 year of storage in ethanol at −20°C.

[^{3}H]ETB-TBOA Binding to Rat Brain Crude Membranes. First, we characterized [^{3}H]ETB-TBOA binding to

rat brain crude membranes, which contain both glial and neuronal membranes. This preparation is expected to reflect the characteristics of the endogenously expressed transporters and is eminently suitable for the initial screening of novel ligands. At a concentration of 10 nM, specific binding was linear with respect to membrane concentration over the range of 5 to 50 μg protein/well (data not shown). [^{3}H]ETB-TBOA rapidly associated with crude membranes (Fig. 2A). Equilibrium was reached within 5 min at room temperature, and specific binding was sustained for more than 120 min. Dissociation of more than 90% of bound [^{3}H]ETB-TBOA by the addition of 10 μM TFB-TBOA occurred within 10 min. Although only 30 and 85% of bound radioactivity was dissociated after 30 min in the presence of 1 or 10 mM L-glutamate, respectively, dissociation reached equilibrium within 10 min (Fig. 2B). With increasing concentrations of [^{3}H]ETB-TBOA saturable-specific binding was observed with a K_d of 29.5 ± 3.7 nM and a B_{max} of 41.1 ± 6.8 pmol/mg protein (Fig. 3, A and B). Nonspecific binding, determined by the addition of 10 μM TFB-TBOA, increased linearly and accounted for approximately 15% of total radioactivity bound at 10 nM radioligand and approximately 30% at 100 nM. [^{3}H]ETB-TBOA binding was displaced by EAAT substrates such as L-glutamate, L-aspartate, and D-aspartate (Fig. 3C). The affinity of L-aspartate and D-aspartate was almost comparable, but that of D-glutamate was lower than L-glutamate. Neither iGluR agonists [α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, *N*-methyl-D-aspartate, and (2*S*,1′*R*,2′*S*)-2-(2-carboxycyclopropyl)glycine] (CCG-IV) nor mGluR agonists [(1*S**,3*R**)-1-aminocyclopentane-1,3-dicarboxilic acid and (2*S*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)glycine] (DCG-IV) inhibited [^{3}H]ETB-TBOA binding to rat brain crude membranes, even at agonist concentrations of 1 mM (data not shown).

Saturation Experiments Using EAAT-Expressing Cell Membranes. Next, we characterized the binding of [^{3}H]ETB-TBOA to each EAAT subtype expressed on COS-1 cells. These experiments used 30 to 100 μg of protein/well. No specific binding was observed in membranes prepared from vector-transfected cells. However, saturable-specific binding was observed in EAAT-expressing cell membranes (Fig. 4A). Nonspecific binding accounted for approximately 5 to 15% of total radioactivity at the concentrations used in the displacement assays (10–20 nM), with the exception of EAAT3-expressing cell membranes, in which the specific binding was less than the other subtypes, reflecting the low affinity of ETB-TBOA for EAAT3. Curve-fitting of saturation experiment data and linear Scatchard plots (Fig. 4B) revealed a one-component binding site for each EAAT subtype, although the K_d and B_{max} values varied (Table 1). The K_d values of

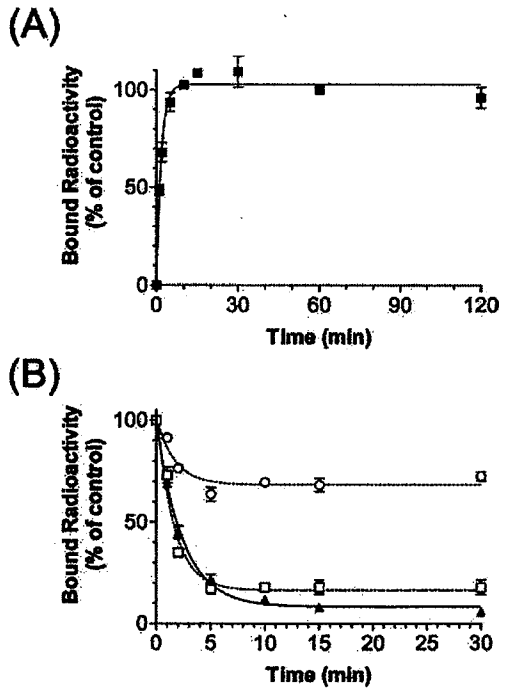

Fig. 2. [^{3}H]ETB-TBOA binding to and dissociation from rat brain crude membranes. A, association of 10 nM [^{3}H]ETB-TBOA at room temperature. Bound radioactivities were normalized to the value at 60 min. B, dissociation of 10 nM [^{3}H]ETB-TBOA in the presence of 1 mM L-glutamate (○), 10 mM L-glutamate (□), or 10 μM TFB-TBOA (▲) after a 30-min association. Data represent the mean ± S.E.M. from three separate experiments.

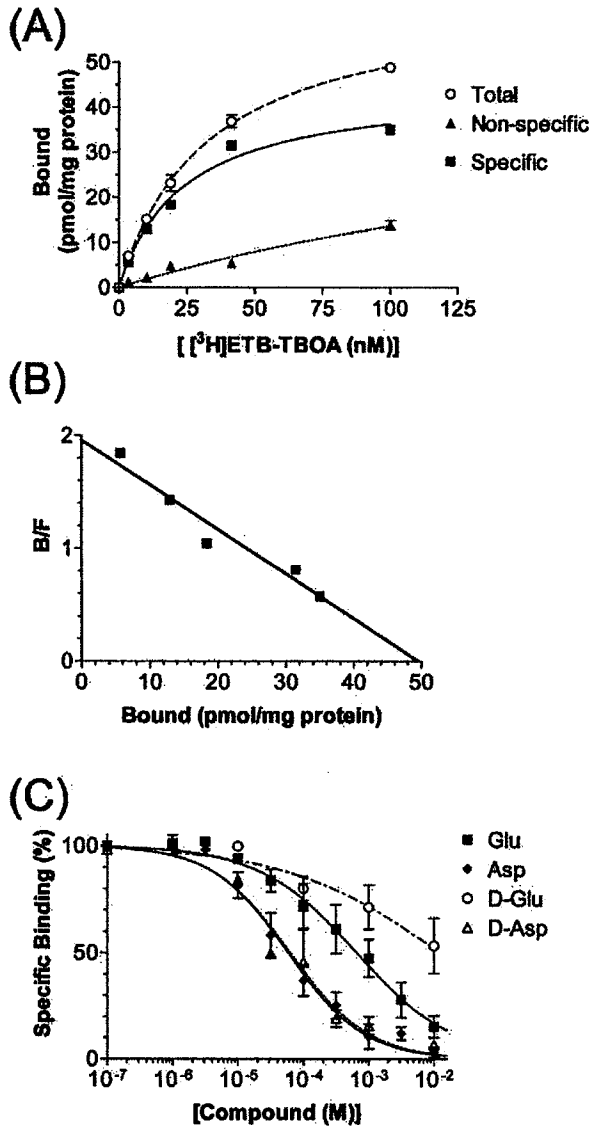

Fig. 3. [^{3}H]ETB-TBOA binding to rat brain crude membranes. A, representative saturation plot; B, its Scatchard analysis. C, inhibition of [^{3}H]ETB-TBOA binding by EAAT substrates. Nonspecific binding was determined in the presence of 10 μM TFB-TBOA. Data represent mean ± S.E.M. of at least three separate experiments.

EAAT1,2,4,5 were 10 to 25 nM, whereas that of EAAT3 was 10-fold higher than the other subtypes.

Ion Dependence. Because transport of glutamate by EAATs is Na^+/K^+-dependent, the ion dependence of [^{3}H]ETB-TBOA binding was investigated using EAAT2-expressing cell membranes. Specific binding completely disappeared in ChCl buffer in which NaCl was replaced by choline chloride (Fig. 5A). When the Na^+ concentration was in-

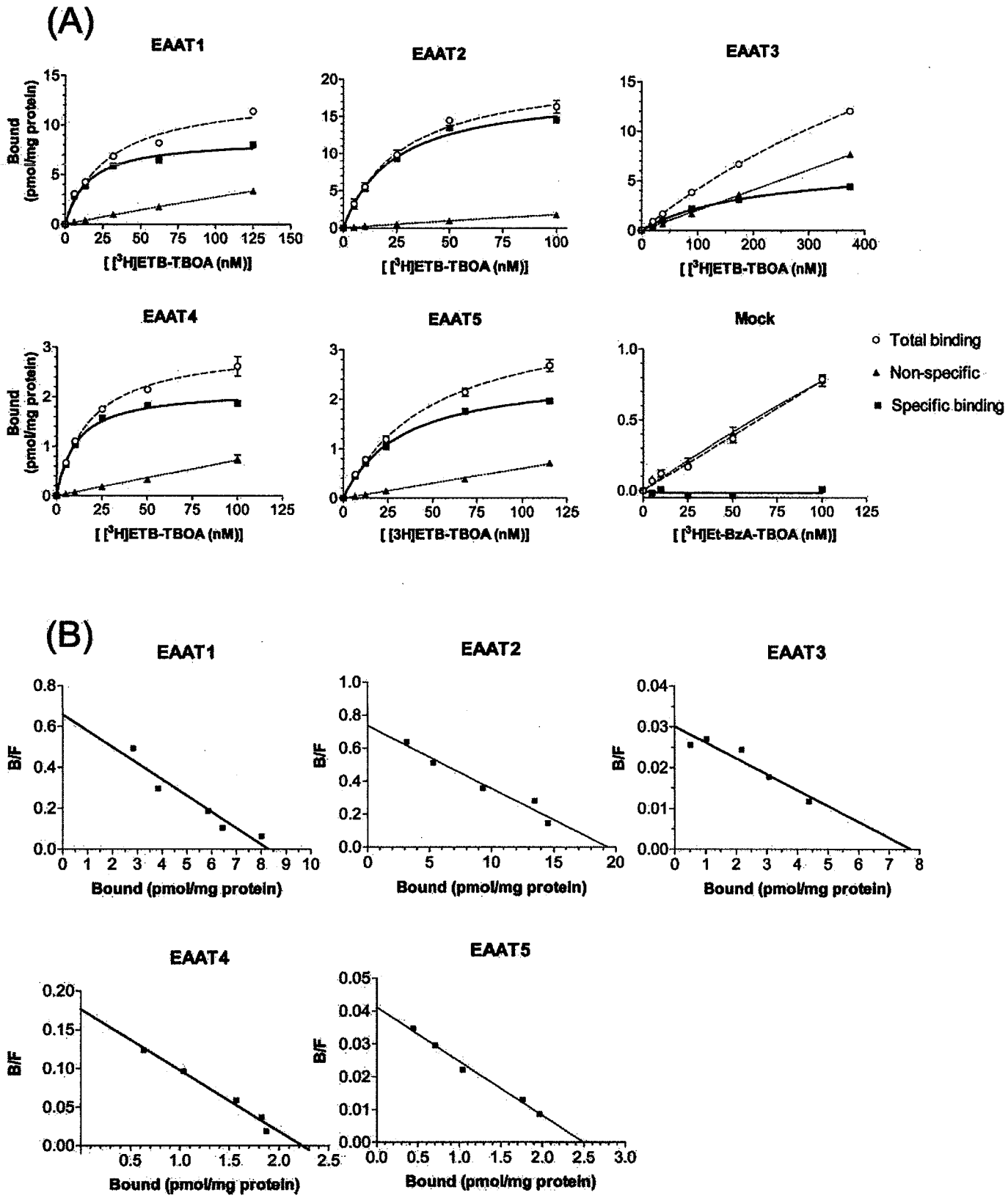

Fig. 4. [^{3}H]ETB-TBOA binding to EAAT-expressing COS-1 cell membranes. A, representative saturation plots. Specific binding was detected in EAAT-expressing cell membranes but not in vector-expressing cell membranes (mock). B, Scatchard analysis of the saturation data.

creased, the specific binding increased, and the K_d values decreased. To determine the stoichiometry of Na^+ binding in the binding process, the Na^+ concentration-dependence of [^{3}H]ETB-TBOA binding was examined (Fig. 5B). Specific binding at each Na^+ concentration was normalized to the value at 120 mM Na^+ (100%). The Hill coefficient determined from a Hill plot of the data were 1.10 ± 0.03 (r^2 = 0.99) and the K_m was 53.3 ± 5.5 mM when the dose-response curve was fitted to a single binding-site model. Other ion dependencies were compared between the [^{3}H]ETB-TBOA binding assay and the [^{14}C]glutamate uptake assay (Fig. 6). Replacement of Na^+ by K^+ also resulted in complete loss of the binding, whereas replacement by Li^+ retained approximately half of the specific binding. It is interesting that [^{14}C]glutamate uptake by COS-1 cells in LiCl buffer was greatly reduced (<10% compared with NaCl buffer). In the presence of Na^+, [^{3}H]ETB-TBOA binding was independent of Cl^- and K^+ ions as shown by binding in chloride-free buffer or potassium-free buffer. The uptake of [^{14}C]glutamate was slightly reduced in the absence of Cl^- or K^+ but not to the extent measured in Na^+-free buffers. There were no differences in cell viability in the different buffers during the uptake assay.

TABLE 1

K_d and B_{max} values of [^{3}H]ETB-TBOA binding to EAAT-expressing COS-1 cell membranes and rat brain crude membranes

Values were determined from the Scatchard analysis. Data are the mean ± S.E.M. from n = 3 to 7 experiments.

	n	K_d	B_{max}
		nM	*pmol/mg protein*
EAAT1	4	15.5 ± 1.1	14.3 ± 9.7
EAAT2	7	16.2 ± 2.5	13.6 ± 3.6
EAAT3	3	320 ± 44	18.9 ± 7.6
EAAT4	3	11.4 ± 0.7	2.6 ± 0.5
EAAT5	4	24.8 ± 5.4	3.6 ± 0.6
Rat brain crude membranes	7	29.5 ± 3.7	41.1 ± 6.8

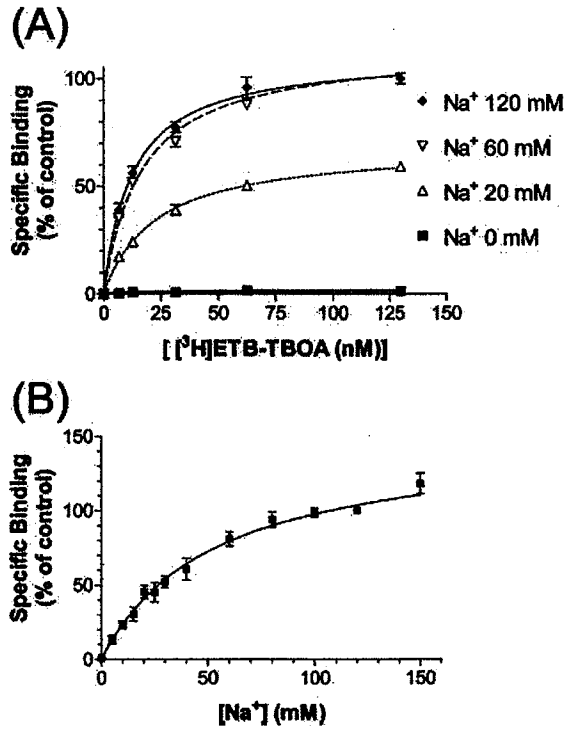

Fig. 5. Sodium ion-dependence of [^{3}H]ETB-TBOA binding to EAAT2-expressing cell membranes. A, saturation plot of [^{3}H]ETB-TBOA binding in various Na^+ ion concentrations. Specific binding was presented as a percentage relative to 125 nM [^{3}H]ETB-TBOA binding in 120 mM NaCl buffer for 30 min. B, specific binding of 20 nM [^{3}H]ETB-TBOA in various Na^+ concentrations. Specific binding at each concentration was normalized to the amount bound at 120 mM Na^+ (100%). Data represent the mean ± S.E.M. of at least three independent experiments (n = 3–15).

Displacement Assay. The effects of other EAAT inhibitors were tested on each subtype (Fig. 7). The K_i values for each compound are summarized in Table 2. Both substrate-type inhibitors (L-*trans*-pyrrolidine-2,4-dicarboxylic acid and CCG-III) and blocker-type inhibitors [L-TBOA, ETB-TBOA, (2*S*,3*S*)-3-{3-[4-methoxybenzoylamino]benzyloxy}aspartate (PMB-TBOA), and TFB-TBOA] displaced [^{3}H]ETB-TBOA binding. For EAATs1–3, the rank order of inhibition was similar to the uptake assay, with the exception that the K_i value of glutamate for EAAT3 was unexpectedly high. The most potent inhibitor for all subtypes was TFB-TBOA. The K_i values of TFB-TBOA for EAAT1,2,4,5 were in the low nanomolar range (3.7–14.8 nM), whereas the K_i for EAAT3 was higher (282 nM). These results were consistent with the electrophysiological results from the *X. laevis* oocyte studies (Shimamoto et al., 2004). Dihydrokainate (DHKA), an EAAT2-selective blocker, displaced [^{3}H]ETB-TBOA binding only in EAAT2-expressing cells (K_i = 541 μM) and not in the other subtypes even at concentrations of 1 mM. Although α-aminoadipate and CCG-II selectively inhibited glutamate uptake in the cerebellum (Robinson et al., 1993), these compounds did not affect [^{3}H]ETB-TBOA binding even at concentrations of 1 mM.

Discussion

A binding assay that could evaluate the effects of inhibitors on all five EAAT subtypes would be very useful for the evaluation of ligands. [^{3}H]D-aspartate, [^{3}H]L-aspartate, and [^{3}H](2*S*,4*R*)-4-methylglutamate ([^{3}H]4MG) have been used previously as radioligands for labeling EAAT (Balcar et al., 1995; Scott et al., 1995; Lieb et al., 2000; Aprico et al., 2001, 2004). However, the K_d values of these ligands were in the micromolar range and characterization of binding by these ligands to each subtype has not yet been examined.

In this study, we synthesized [^{3}H]ETB-TBOA as a novel radioligand. [^{3}H]ETB-TBOA rapidly associated with rat brain crude membranes and showed significant specific binding. The radioligand was dissociated by the addition of unlabeled ligands. The saturation curve fit a single binding-site model. The B_{max} value was approximately 40-fold larger than

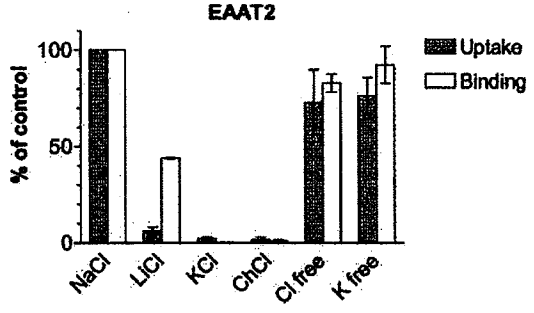

Fig. 6. Effects of ions on [^{3}H]ETB-TBOA binding to EAAT2-expressing cell membranes (□) and [^{14}C]glutamate uptake by EAAT2-expressing cells (▩). Data represent mean ± S.E.M. of at least three separate experiments.

that of iGluRs in rat brain crude membranes (1.1–1.6 pmol/mg of protein) (London and Coyle, 1979; Murphy et al., 1987; Sills et al., 1991), which reflects the fact that EAATs are abundant in brain (Danbolt, 2001). The substrate-specificity in the displacement assays indicates that ETB-TBOA binds competitively to a substrate binding site. In contrast, GluR agonists did not inhibit [^{3}H]ETB-TBOA binding, and ETB-TBOA did not affect the activity of iGluRs, mGluRs, or vesicular glutamate transporters. Thus, the majority of the bound radioactivity is attributable to EAATs.

Significant specific binding was also obtained using individual EAAT subtypes on EAAT-expressing cell membranes. Each subtype showed the presence of a single binding site. The K_d values for EAAT1,2,4,5 were 10 to 25 nM, whereas that for EAAT3 was more than an order of magnitude higher. We have reported previously that EAAT3 was less potently inhibited by (2*S*,3*S*)-3-{3-[4-substituted-benzoylamino]benzyloxy}aspartate than the other subtypes (Shimamoto et al., 2004). The B_{max} values of EAAT4 and EAAT5 were much lower than those of EAATs1–3, which may partly reflect the low uptake by EAAT4 and EAAT5.

Specific binding by [^{3}H]ETB-TBOA was Na^+-dependent and was completely lost in ChCl buffer. The affinity increased with increasing Na^+-concentrations. These results indicate that at least one Na^+ ion binds to the transporter before substrate binding, which is in agreement with a previous report (Watzke et al., 2001), and suggest that the Na^+ ion changes the protein conformation to an active form. It is interesting that the Hill coefficient for the Na^+ dependence of [^{3}H]ETB-TBOA binding in this study was approximately 1 (1.10 ± 0.03), indicating that Na^+ binds to a single class of noncooperative binding sites. In contrast, the Hill coefficients

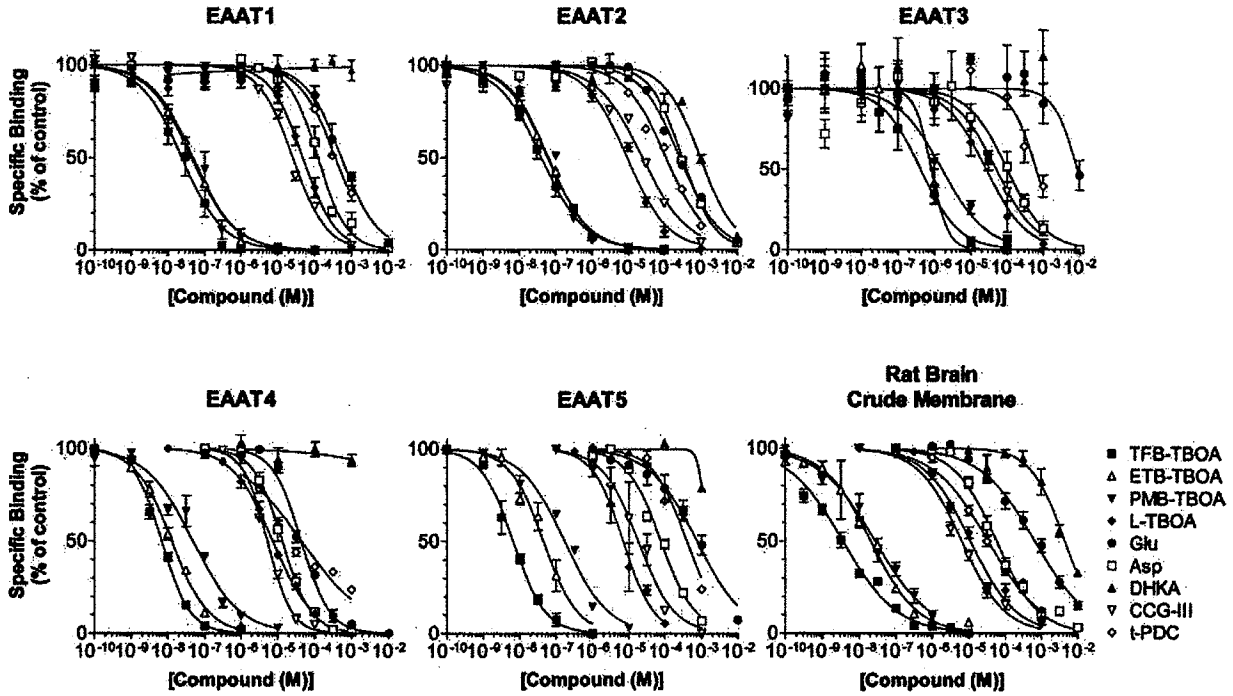

Fig. 7. Displacement of [^{3}H]ETB-TBOA binding by drugs known to interact with EAATs in EAAT-expressing cell membranes and rat brain crude membranes. Specific binding was normalized to the control (100%). Nonspecific binding was determined in the presence of 10 μM TFB-TBOA. Data represent mean ± S.E.M. of at least three separate experiments. The concentration of [^{3}H]ETB-TBOA used and the K_i values of inhibitors for each preparation are summarized in Table 2.

TABLE 2

K_i values of various inhibitors of [^{3}H]ETB-TBOA binding

K_i values were determined from the IC_{50} values determined in Fig. 7. Data are the mean ± S.E.M.

	EAAT1	EAAT2	EAAT3	EAAT4	EAAT5	Rat Brain Crude Membranes
			μM			
[^{3}H]ETB-TBOA	20 nM	20 nM	100 nM	10 nM	20 nM	10 nM
TFB-TBOA	0.0096 ± 0.0040	0.015 ± 0.0063	0.28 ± 0.11	0.0039 ± 0.0003	0.0037 ± 0.0004	0.0027 ± 0.0004
ETB-TBOA	0.021 ± 0.0015	0.030 ± 0.0031	0.71 ± 0.21	0.0068 ± 0.0009	0.025 ± 0.0039	0.015 ± 0.0016
PMB-TBOA	0.021 ± 0.0047	0.045 ± 0.0071	1.2 ± 0.30	0.026 ± 0.0042	0.095 ± 0.016	0.020 ± 0.0034
L-TBOA	26 ± 6.2	6.6 ± 0.9	20 ± 3.5	3.9 ± 0.59	3.9 ± 0.61	8.4 ± 1.3
L-Glutamate	259 ± 49	137 ± 17	5065 ± 2830	26 ± 2.5	420 ± 80	490 ± 44
L-Aspartate	47 ± 10	125 ± 8.7	67 ± 1.4	8.5 ± 1.2	44 ± 3.7	44 ± 4.7
t-2,4-PDC	144 ± 44	50 ± 13	347 ± 146	23 ± 2.5	403 ± 82	26 ± 4.0
CCG-II	>1000	>1000	>1000	>1000	>1000	>1000
CCG-III	9.2 ± 0.8	10 ± 2.2	21 ± 1.0	2.8 ± 0.16	11 ± 2.5	4.8 ± 0.7
DHKA	>1000	541 ± 3.0	>1000	>1000	>1000	3324 ± 442
α-Aminoadipate	>1000	>1000	>1000	>1000	>1000	>1000

t-2,4-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid.

for substrate transport (L-glutamate or D-aspartate), measured by uptake assays or electrophysiological studies, were reported to be 1.9 to 3.3, which most likely reflects the requirement for two to three Na^+ ions to complete the uptake process (Klöckner et al., 1993; Sugawara et al., 1998; Kimmich et al., 2001). These results suggest that Na^+ binding in the ligand binding step is different from Na^+ binding in the substrate-uptake process. ETB-TBOA is a blocker and is involved only in the ligand binding step. Additional Na^+ ion binding steps would be necessary for subsequent steps in the transport process. Moreover, similar to previous observations, Li^+ ions could partly substitute for Na^+ ions (Borre and Kanner, 2001; Larsson et al., 2004). In contrast, K^+ could not be replaced by Na^+, probably because of its larger ion radius. K^+ was not essential for substrate binding, although it is indispensable for uptake. In addition, the absence of Cl^- ion did not affect binding.

EAAT inhibitors displaced [^{3}H]ETB-TBOA in EAAT-expressing cell membranes and in rat brain crude membranes. The K_i values were very similar to the IC_{50} values reported previously in uptake assays using EAATs1–3 (Arriza et al., 1994; Shimamoto et al., 1998), whereas some values were higher than those obtained from [^{3}H]4MG or [^{3}H]D-aspartate binding in rat brain membrane homogenates or astrocytic membrane homogenates (Aprico et al., 2001, 2004). This discrepancy may be due to the fact that the concentrations of [^{3}H]4MG or [^{3}H]D-aspartate (40 nM) in the previous studies were much lower than the K_d values of the compounds (6.0 and 15.0 μM, respectively), whereas, in the present experiments, the concentrations (10–100 nM) of [^{3}H]ETB-TBOA were similar to the K_d values. The overall rank order of activity was similar to previous uptake assays and electrophysiological studies, with the exception that the affinity of L-glutamate for EAAT3 was unexpectedly low. The K_i value of L-glutamate for EAAT3 was more than 5 mM, which is approximately 180 times larger than the K_m value obtained from the electrophysiological assay (28 μM), whereas L-aspartate showed a K_i value (67 μM) that would be reasonably expected from its K_m value (24 μM) (Arriza et al., 1994). Because the affinity of [^{3}H]ETB-TBOA for EAAT3 is low, higher-affinity radiolabeled ligands for EAAT3 would be necessary to further elucidate the molecular basis for the discrepancies. Although the differences were not as great as that seen with EAAT3, L-aspartate showed higher affinity than L-glutamate in all subtypes.

The binding site of EAATs recognizes the folded form of L-glutamate or the anti-form of aspartates (Bridges and Esslinger, 2005), and many analogs mimicking these conformations have been designed. One of the folded-type glutamate analogs, L-CCG-III (Nakamura et al., 1993), inhibited [^{3}H]ETB-TBOA binding to all subtypes with moderate potency (low micromolar concentration). The other isomers, L-CCG-I, II, and IV, did not markedly inhibit [^{3}H]ETB-TBOA binding even at concentrations of 1 mM. Evaluation of subtype selective inhibitors is one of the important roles of the assay. DHKA, a well-known EAAT2-selective blocker, displaced [^{3}H]ETB-TBOA binding to EAAT2 only at a concentration of 1 mM, although it was less potent in our preparations than in other reports (Shimamoto et al., 1998). Glutamate uptake into synaptosomes is inhibited by DHKA, suggesting EAAT2 is primarily responsible. The inhibition by DHKA of [^{3}H]ETB-TBOA binding in rat crude membranes was, however, weaker than inhibition of binding to EAAT2-expressing cell membranes. EAAT2 is responsible for 30 to 50% of [^{3}H]4MG binding in rat brain membranes (Aprico et al., 2001). Recently EAAT2-preferring blockers have been reported (Dunlop et al., 2003, 2005; Bunch et al., 2006), but their effects on EAAT4 and EAAT5 are unknown. Characterization of these compounds in the binding assay developed here may further elucidate their specificity. Although (2*S*,1′*R*,2′*R*)-2-(2-carboxycyclopropyl)glycine (CCG-II) and α-aminoadipate were reported to inhibit glutamate uptake in the cerebellum (Robinson et al., 1993) and α-aminoadipate was used as a substrate for neuronal transporters in cerebellum preparations, they did not bind to EAAT4 even at concentrations of 1 mM. Because α-aminoadipate also inhibits the glutamate-cystine antiporter, its effects on EAATs need to be verified in electrophysiological studies.

The most potent EAAT inhibitor tested here was TFB-TBOA (Shimamoto et al., 2004). The K_i values of TFB-TBOA for [^{3}H]ETB-TBOA binding to EAATs1–3 (9.6, 15, and 282 nM, respectively) were similar in magnitude to the IC_{50} values determined by the glutamate uptake assay (22, 17, and 300 nM, respectively). TFB-TBOA also showed a high affinity for EAAT4 and EAAT5 (3.9 and 3.7 nM, respectively). As expected from the results of the uptake assays, ETB-TBOA and PMB-TBOA were slightly less potent in binding to EAATs1–3 than TFB-TBOA. It is interesting that the affinity of PMB-TBOA for EAAT5 was markedly lower than that of TFB-TBOA. The binding affinity for EAAT5 was strongly affected by substituents. Recently, Takayasu et al. (2006) reported that PMB-TBOA blocked the transport current evoked by stimulation of the climbing fiber in Bergmann glia approximately 10-fold more potently than in Purkinje cells. EAAT4 is preferentially involved in clearing glutamate from climbing fiber-Purkinje cell synapses (Huang et al., 2004; Takayasu et al., 2005). In the present study, however, the affinity of PMB-TBOA for EAAT4 was comparable with its affinity for the glial transporters (EAAT1 and EAAT2), suggesting that unknown mechanisms regulate the affinity in vivo. Development of EAAT4-selective inhibitors would elucidate these discrepancies.

In conclusion, the [^{3}H]ETB-TBOA binding assay will provide useful pharmacological information about EAATs. The binding assay using frozen brain crude membranes is much easier to conduct than the uptake assay using freshly prepared synaptosomes, and thus, it is applicable to high-throughput screening to find novel EAAT ligands or to examine the side effects of drugs on EAATs. [^{3}H]D-aspartate has been used for comparison of synaptosomal preparations between nondemented control and diseased brain samples (Scott et al., 1995), and [^{3}H]L-aspartate has been used for autoradiographic studies (Lieb et al., 2000). Because the binding affinity of [^{3}H]ETB-TBOA is much higher than binding affinities of the aspartates and [^{3}H]ETB-TBOA does not interact with other proteins, similar applications are possible. In addition, the lack of a simple assay system to evaluate the compounds binding to EAAT4 and EAAT5 has prevented the full characterization of subtype-selective inhibitors. Using cell membranes expressing each EAAT subtype, this binding assay has enabled direct and quantitative comparisons among all subtypes. Thus, [^{3}H]ETB-TBOA should serve as a useful tool to test newly developed subtype-selective EAAT ligands and will result in a better understanding of

synaptic transmission mechanisms and the etiology of neuronal disorders.

Acknowledgments

We thank Professor E. Gouaux (Oregon Health and Science University, Portland, OR) for providing useful information about sodium coupling. We thank Professor T. Ueda and Dr. D. Bole (University of Michigan, Ann Arbor, MI) for conducting the vesicular glutamate uptake assay. We also thank Professor S. G. Amara (University of Pittsburgh, Pittsburgh, PA) for providing MDCK cells expressing EAAT2 or EAAT3 and cDNA of pOTV-EAAT5, Professor S. Nakanishi (Osaka Bioscience Institute, Osaka, Japan) for providing Chinese hamster ovary cells expressing mGluRs, and T. Maeda (Suntory Institute for Bioorganic Research, Osaka, Japan) for technical assistance.

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